Comprehensive Analysis of microRNA Profiles in Organoids Derived from Human Colorectal Adenoma and Cancer

Hiroshi Nagai^a Masatake Kuroha^a Tomoyuki Handa^a Hideaki Karasawa^b
Shinobu Ohnuma^b Takeo Naito^a Rintaro Moroi^a Yoshitake Kanazawa^a
Hisashi Shiga^a Shin Hamada^a Yoichi Kakuta^a Takeshi Naitoh^b
Yoshitaka Kinouchi^c Tooru Shimosegawa^a Atsushi Masamune^a

^aDivision of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan; ^bDepartment of Gastrointestinal Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan; ^cHealth Administration Center, Center for the Advancement of Higher Education, Tohoku University, Sendai, Japan

Keywords

Adenoma-carcinoma sequence · Exosome · Extracellular vesicle · miR-1246 · Tumor microenvironment

Abstract

Introduction: Exosomes are membrane-enclosed nanovesicles, which are increasingly being recognized as important cell communication components for their role in transmitting microRNAs (miRNAs). No previous study has addressed the exosomal miRNA profile in colorectal adenomas (CRAs) because the long-term culture of CRA is challenging. This study aimed to identify the miRNA signature in organoid exosomes derived from human CRA and colorectal cancer (CRC) samples. **Methods:** Organoid cultures were developed from resected colorectal tissues of patients with CRA or CRC undergoing surgery or endoscopic mucosal resection. Exosomes were prepared from the conditioned medium of the organoids. miRNAs were prepared from the exosomes and their source organoids. The miRNA expression profiles were compared using microarray analysis. The impact of alteration of miRNA expression on cell proliferation was examined using miRNA mimics or inhibitors in HT-29 human CRC cells. **Results:** We established 6 organoid lines from CRC and 8 or-

ganoid lines from CRA. Exosomal miRNA signatures were different between the organoids derived from CRA and CRC. Both exosomal and cellular miR-1246 expressions were up-regulated in CRC-derived organoids compared to their expression in CRA-derived organoids. Alteration of miR-1246 expression by the miR-1246 mimic or inhibitor increased or decreased cell proliferation in HT-29 cells, respectively. **Conclusions:** We report for the first time the miRNA profiles of exosomes in CRA- and CRC-derived organoids. The upregulation of miR-1246 might play a role in increased cell proliferation in the process of CRA-carcinoma transition.

© 2021 The Author(s)
Published by S. Karger AG, Basel

Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide [1]. Identification of the colorectal adenoma (CRA)-carcinoma transition with its corresponding genetic and epigenetic alterations has significantly increased our knowledge of the etiopathogenesis of CRC. Recently, a novel 3-dimensional organoid culture of CRC was developed and used to model tumorigenesis and drug resistance [2, 3]. In organoid cultures, cancer

stem cells are maintained in a microenvironment by using a cocktail of niche factors, including noggin, R-spondin 1, and epidermal growth factor, supplemented in the medium. It is known that the niche factors become unnecessary as the CRC progresses to malignancy, suggesting an important role of the microenvironment in colon cancer progression [4].

MicroRNAs (miRNAs) are small (19–23 nucleotide) noncoding RNAs that regulate gene expression by targeting the 3' untranslated region of the target messenger RNA [5]. miRNAs are involved in the regulation of cancer cell characteristics and the maintenance of the microenvironment. For example, miR-135b is upregulated in CRC and is associated with tumor progression and poor clinical outcome [6]. miR-34a is a cell fate determinant in early-stage dividing colon cancer stem cells, and it functions through Notch signaling [7]. Recently, miRNAs were shown to exist stably in the extracellular space by forming extracellular vesicles termed exosomes. Exosomes are 40- to 100-nm nanosized vesicles that contain diverse host cell-derived bioactive molecules, such as proteins, lipids, and miRNAs [8–10]. Exosomes can be secreted from various cell types into the extracellular space, where they can be taken up by neighboring and distant cells and subsequently can modulate the functions of the recipient cells. Exosomes secreted from CRC cells trigger the activation of fibroblasts to α -smooth muscle actin-positive myofibroblast-like phenotype, leading to the development of a cancer-conditioned microenvironment [11]. Exosomal miR-210 secreted from CRC cells influences the adhesion of neighboring metastatic cells [12]. However, no previous studies have addressed miRNA signatures in exosomes derived from CRAs due to the difficulty of the long-term culture of CRAs. This study aimed to identify the miRNA signature in organoid exosomes derived from human CRA and CRC samples.

Material and Methods

Materials

The mouse anti-CD9 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse anti-CD63 antibody was obtained from Becton Dickinson (Franklin Lakes, NJ, USA). The peroxidase-linked secondary antibody was purchased from GE Healthcare (Chicago, IL, USA). Cell culture plates, dishes, and culture inserts were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The reagents and instruments for qRT-PCR were purchased from Applied Biosystems (Foster City, CA, USA). All other reagents were obtained from Gibco (Grand Island, NY, USA) unless specifically indicated.

Organoid Culture and Passage

Fourteen human colorectal tissues (6 CRCs and 8 CRAs) were isolated from the resected colorectal tissues of patients undergoing surgery or endoscopic mucosal resection at Tohoku University Hospital between June 2016 and March 2018, under the approval of the Ethics Committee of Tohoku University, Graduate School of Medicine (article#: 2015-4-011). All patients provided written informed consent. Dissected tissues were pelleted and resuspended into the extracellular matrix. We prepared the human organoid culture medium, as described previously [13] with slight modifications. In brief, niche factors consisting of epidermal growth factor, recombinant murine noggin (PeproTech, Rocky Hill, NJ, USA), A83-01 (Wako Chemical, Osaka, Japan), and SB209120 (Cayman Chemical, Ann Arbor, MI, USA) were added to Advanced DMEM/F12 medium with GlutaMAXTM supplement, B27 supplement, Gastrin-I (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. The medium was replaced with fresh medium every 2 or 3 days until the organoid cultures became confluent.

Cell Culture and Transfection

The human colon cancer line HT-29 was obtained from the American Type Culture Collection and was maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO₂. We used miR-1246 mimic (#MC13182), miR-1246 inhibitor (#MH13182), and mirVanaTM miRNA mimic/inhibitor negative control #1 from Ambion (Austin, TX, USA). These RNA oligonucleotides were transiently transfected into HT-29 cells using LipofectamineTM RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Isolation of Exosomes Released from Organoids

Exosomes were prepared from organoids, as previously described [14, 15] with slight modifications. In brief, organoids were cultured in 24-well culture plates (1 × 10⁵ cells/well), and the conditioned medium was harvested and centrifuged at 300 g for 10 min, followed by centrifugation at 2,000 g for 30 min to eliminate cells and debris. This was followed by ultracentrifugation at 100,000 g at 4°C for 70 min to pellet the exosomes. The supernatant was discarded, and the resulting pellets were carefully resuspended in PBS followed by ultracentrifugation at 100,000 g at 4°C for 70 min. Following the removal of the supernatant, the pelleted exosomes were resuspended in PBS. The isolated exosomes were stored at –80°C until use.

Assessment of Morphology and Size Distribution

Isolated exosomes were evaluated for their morphology and size distribution by transmission electron microscopy, as previously described [15]. Exosomal pellets were fixed in 2% (v/v) glutaraldehyde, rinsed, and post-fixed in 2% osmium tetroxide for 2 h. Then, the pellets were rinsed with distilled water, dehydrated through a graded series of ethanol, and embedded in an epoxy resin at 60°C. Ultrathin sections were prepared using an ultramicrotome (Leica EM UC7; Leica Microsystems Japan, Tokyo, Japan), mounted on copper grids, and counterstained with uranyl acetate and lead citrate. A Hitachi H-7600 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan) was used to acquire images. ImageJ software (<https://imagej.nih.gov/ij/>) was used to measure the size of the exosomes in the captured images.

The exosome amount and size distribution were also analyzed using a NanoSight LM10 microscope (NanoSight Ltd., Amesbury, UK). Samples were analyzed 3 times, each for 30 s.

Western Blotting

The isolated exosomes were lysed and fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Then, they were transferred to PVDF membranes (GE Healthcare), and the membranes were incubated with mouse anti-CD63 antibody for 1 h at room temperature. After incubation with the peroxidase-conjugated anti-mouse IgG antibody, proteins were visualized using the Prime™ ECL Western Blotting Detection Reagent (GE Healthcare). The expression of CD9 was determined in a similar manner.

RNA Extraction and miRNA Microarrays

Total RNAs, including miRNAs, were extracted from patient-derived organoids and isolated exosomes using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and quality were evaluated using a Bioanalyzer 2100 Expert (Agilent Technologies, Santa Clara, CA, USA) in conjugation with the RNA 6000 Pico Kit (Cat. No. 5067-1513; Agilent Technologies).

The Agilent miRNA microarray system (Human miRNA microarray Release 21.0, Agilent; Agilent Technologies), containing probes for 2,588 human miRNAs, was used to identify differentially expressed cellular and exosomal miRNAs between CRC-derived organoids and CRA-derived organoids. The arrays were scanned; the data were extracted and analyzed using Agilent Feature Extraction software and Agilent GeneSpring software. miRNAs with $|\log_2$ fold change| > 1 were identified as differentially expressed miRNAs.

Quantitative Real-Time PCR

qRT-PCR was performed using TaqMan™ Advanced miRNA assays. The assay IDs of the probes were #477881 for hsa-miR-1246, #480541 for hsa-miR-7107-5p, #480931 for hsa-miR-8485, #480099 for hsa-miR-5100, #479561 for hsa-miR-1273g-3p, #478096 for hsa-miR-4286, and #489882 for hsa-miR-4649-3p. PCR was performed at 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s using the StepOne Plus™ 7300 Real-Time PCR instrument. A comparative Ct method was used for the quantification of the expression of each miRNA; then, the expression levels were normalized to those of hsa-miR-4649-3p because it was detectable in all samples and its normalized intensities were not significantly different between CRC- and CRA-derived exosomes. Data were collected from 3 independent examinations.

Cell Proliferation Assay

HT-29 cells were seeded in 96-well plates (2×10^3 /well), and cell proliferation was determined at 24, 48, and 72 h using the CellTiter 96® AQueous One Solution Cell Proliferation assay (MTS assay; Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

Data from at least 3 independent experiments were analyzed and were represented as mean $\pm$ SD. All statistical analyses were conducted using JMP software (SAS Institute, Cary, NC, USA). The differences between the 2 groups were analyzed using the unpaired *t* test. A *p* value <0.05 was considered statistically significant, and *p* < 0.01 was deemed highly significant.

Table 1. Clinical characteristics of enrolled patients with CRC and CRA

Patients No.	Age, years	Sex	Location	Stage
CRC				
1	81	F	Right colon	I
2	58	M	Right colon	I
3	71	M	Right colon	II
4	91	F	Rectum	I
5	76	M	Right colon	IIIa
6	64	M	Rectum	I
CRA				
1	62	F	Rectum	na
2	61	M	Rectum	na
3	78	M	Right colon	na
4	67	M	Right colon	na
5	63	M	Left colon	na
6	58	M	Right colon	na
7	71	F	Right colon	na
8	75	M	Left colon	na

CRC, colorectal cancer; CRA, colorectal adenoma.

Results

Successful Isolation of Exosomes from Patient-Derived Organoids

We established 6 organoid lines from CRC and 8 organoids from CRA. The clinicopathological characteristics of the enrolled patients with CRC and CRA are presented in Table 1. Paraffin sections of the organoids indicated that the established organoids imitated the original structures as a budding form, which could be seen with H-E staining (Fig. 1a). Exosomes were isolated from the conditioned medium of organoids by ultracentrifugation (Fig. 1b). Under transmission electron microscopy, the size of the vesicles was estimated to be around 100 nm (Fig. 1c), which corresponded to the results of previous studies [8, 10]. The nanoparticle-tracking analyzer showed that the mode size of the vesicles in isolated exosomes was 103.5 ± 3.2 nm (Fig. 1d). The exosomal markers CD9 and CD63 were positive for isolated exosomes but negative for the control ultracentrifuged medium without organoids (Fig. 1e).

Microarray Analysis Revealed Different miRNA Expression Profiles between CRC and CRA

Next, we isolated miRNAs from organoids and exosomes released from organoids derived from CRC and CRA (Fig. 2a). We confirmed that the isolated exosomal miRNA did not contain ribosomal RNA (Fig. 2b, c). Mi-

Fig. 1. Isolation of exosomes from organoids. Exosomes were isolated from the CM of the organoids. **a** Top: images of the primary tumor. Endoscopic imaging (left side) and H-E stained specimen (right side). Bottom: images of the established organoid cell line. Optical microscopic imaging (left side) and H-E-stained specimen (right side). Each scale bar represents 100 μ m. **b** Procedure for the isolation of exosomes released from the organoids by ultracentrifugation. **c** Electron microscopy imaging of isolated exosomes. Original magnification: $\times 33,000$. Scale bar represents 100 nm. **d** Size distribution of isolated exosomes was analyzed using a NanoSight LM10 microscope. **e** Expression of exosome-specific markers, CD63 and CD9, in isolated exosomes and the control medium without organoids, was examined by Western blotting; CM, conditioned medium.

croarray analysis was conducted for each of the CRC-derived organoids (CRC1, CRC2, and CRC3) and CRA-derived organoids (CRA1, CRA2, and CRA3). For cellular miRNAs, the expression of 4 miRNAs was upregulated, while 10 miRNAs were downregulated in the CRC-derived organoids when compared to those in CRA-derived organoids (Fig. 2d; Table 2). In addition, for exosomal miRNAs, the expression of 3 miRNAs was upregulated, while that of 12 miRNAs was downregulated in CRC-derived organoids when compared to those in CRA-derived organoids (Fig. 2e; Table 2). The expression of 2 miRNAs (mir-1246 and mir-7107-5p) was upregulated in CRC-derived organoids, both in the cellular compartments and exosomes (Fig. 2f). However, no miRNA was downregulated in both the cellular compartments and the exosomes.

Next, we examined to what extent the miRNA profiles of the cellular compartments and exosome in CRA- and

CRC-derived organoids matched. Of the 3 CRA-derived organoids, 196 miRNAs were expressed commonly in both the cellular compartments and exosomes; 169 miRNAs, only in the cellular compartments; and 20 miRNAs, only in the exosomes (Fig. 2g). Of the 3 CRC-derived organoids, 167 miRNAs were expressed commonly in both the cellular compartments and exosomes; 198 miRNAs, only in the cellular compartments; and 26 miRNAs, only in exosomes (Fig. 2h). The list of miRNAs is provided in online suppl. File; for all online suppl. material, see www.karger.com/doi/10.1159/000513882.

Exosomal miR-1246 Was Upregulated in CRC-Derived Organoids

We validated the miRNA expression changes identified by the microarray assay using RT-PCR (Fig. 3). The exosomal miRNAs from the 6 CRC- and 8 CRA-derived

Increased miRNAs in CRC derived organoids

Exosomal and cellular miRNA of CRA-derived organoids

Exosomal and cellular miRNA of CRA-derived organoids

organoids were evaluated. Among the top 3 upregulated or the 3 downregulated miRNAs, only miR-1246 was significantly upregulated in the exosomes of the CRC-derived organoids when compared to those of the CRA-derived organoids ($p = 0.0496$). The expression of miR-8485 appeared to be upregulated, but this change was not statistically significant ($p = 0.189$). Similarly, the expression of miR-5100 appeared to be downregulated, but this change was not statistically significant ($p = 0.091$).

miR-1246 Plays a Role in Proliferation in HT-29 Cells

Cell proliferation was analyzed by the MTS assay to clarify the role of the upregulated miR-1246 in CRC. As several miR-1246 mimics could not effectively increase miR-1246 expression in the organoid culture cells, we examined the cell proliferation using human CRC HT-29 cells. HT-29 cells were transfected with the miR-1246 mimic/inhibitor. After 48 h, the expression of miR-1246 was higher in cells transfected with

Table 2. Differentially expressed miRNAs detected using microarray

Carcinoma/adenoma			Adenoma/carcinoma		
systematic name	log ₂ FC	p value	systematic name	log ₂ FC	p value
<i>Exosomal miRNA</i>					
hsa-miR-1246	2.48	0.13	hsa-miR-5100	3.35	0.11
hsa-miR-8485	1.27	0.08	hsa-miR-4286	3.06	0.04
hsa-miR-7107-5p	1.09	0.53	hsa-miR-1273g-3p	2.89	0.20
			hsa-miR-6089	2.53	0.34
			hsa-miR-7975	2.31	0.02
			hsa-miR-1260b	2.29	0.02
			hsa-miR-4459	2.20	0.43
			hsa-miR-8069	2.01	0.30
			hsa-miR-6749-5p	1.99	0.17
			hsa-miR-1260a	1.98	0.11
			hsa-miR-5739	1.98	0.22
			hsa-miR-7977	1.91	0.07
<i>Cellular miRNA</i>					
hsa-miR-1246	1.49	0.21	hsa-miR-210-3p	2.42	0.12
hsa-miR-4459	1.23	0.38	hsa-miR-23b-3p	1.37	0.23
hsa-miR-4741	1.18	0.54	hsa-miR-27-3p	1.36	0.18
hsa-miR-7107-5p	1.13	0.56	hsa-miR-320d	1.36	0.34
			hsa-miR-320b	1.35	0.28
			hsa-miR-320e	1.25	0.33
			hsa-miR-320a	1.13	0.25
			hsa-miR-192-3p	1.10	0.27
			hsa-miR-200b-3p	1.05	0.17
			hsa-miR-200a-3p	1.04	0.22
miRNA, microRNA.					

Fig. 2. Identification of CRC-specific exosomal and cellular miRNA of the organoids. **a** Procedure for the identification of exosomal and cellular miRNAs differentially expressed between CRC- and CRA-derived organoids. **b, c** Total cellular and exosomal RNAs were prepared from CRC- and CRA-derived organoids, and their integrity and quality were examined using a bioanalyzer system. Exosomal RNA did not contain ribosomal RNAs. **d, e** The Agilent's miRNA microarray was used to compare the miRNA expression profiles. miRNAs with $|\log_2$ fold change| > 1 were selected as differentially expressed. Exosomal miRNAs were prepared from CRC- and CRA-derived organoids. **d** Three miRNAs were upregulated, while 12 miRNAs were

downregulated in CRC. **e** Among cellular miRNAs, 4 were up-regulated, and 10 were downregulated in CRC-derived organoids. **f** Both cellular and exosomal miR-1246 and miR-7107-5p were upregulated in CRC-derived organoids. **g** In CRA-derived organoids, 196 miRNAs were expressed commonly in both the cellular compartments and exosomes; 169 miRNAs, only in the cellular compartments; and 20 miRNAs, only in exosomes. **h** In CRC-derived organoids, 167 miRNAs were expressed commonly in both the cellular compartments and exosomes; 198 miRNAs, only in the cellular compartments; and 26 miRNAs, only in exosomes. miRNA, microRNA; CRC, colorectal cancer; CRA, colorectal adenoma.

Fig. 3. Expression levels of the 6 selected exosomal miRNAs by qPCR. Box-and-whisker plots of the expression levels of the 6 selected exosomal miRNAs in an independent set of CRC ($n = 6$) and CRA ($n = 8$). Each data point was normalized to the CRA samples. *: $p < 0.05$ versus control. The data of CRC-derived organoids (CRC1, CRC2, and CRC3) and CRA-derived organoids (CRA1, CRA2, and CRA3), which were analyzed by microarray, are indicated by red circles. miRNA, microRNA; CRC, colorectal cancer; CRA, colorectal adenoma; a-EV, exosomes obtained from CRA-derived organoids; c-EV, exosomes obtained from CRC-derived organoids.

miR-1246 mimics (Fig. 4a) but was lower in cells transfected with miR-1246 inhibitors (Fig. 4b), when compared to the miR-1246 expression in their respective controls. The overexpression of miR-1246 significantly increased the growth of HT-29 cells ($p = 0.019$) (Fig. 4c), whereas the inhibition of miR-1246 significantly suppressed its proliferation ($p = 0.005$) (Fig. 4d) after 72 h, suggesting miR-1246 might have a role in cell proliferation in CRC.

Discussion

In our study, we established patient-derived organoids of CRC and CRA and confirmed the presence of exosomes using transmission electron microscopy and a

nanoparticle-tracking analyzer. In addition, we verified the expression of CD63 and CD9 via Western blotting. Because long-term cultures are needed for the isolation of exosomes from the culture supernatant in vitro, a number of studies have analyzed exosomes derived from cancer cell lines that can survive without niche factors. However, recently, a 3-dimensional culture method mimicking the tissue microenvironment was developed, making it possible to culture patient-derived colorectal tumors for longer periods [2]. While some reports have mentioned the isolation of exosomes from CRC cell lines under a 3-dimensional culture, to our knowledge, this is the first reported analysis of exosomes from patient-derived CRC and CRA using a 3-dimensional culture. Eguchi et al. [16] reported that in exosomes secreted from cell lines, the expression levels of exosomal proteins such as E-cadherin

Fig. 4. Overexpression of miR-1246 increased cell proliferation in HT-29 cells. HT-29 cells were transfected with miR-1246 mimic, miR-1246 inhibitor, or their respective controls. **a, b** qRT-PCR confirmed significant alterations in miR-1246 expression after transfection with miR-1246 mimic (**a**) or miR-1246 inhibitor (**b**) when compared to their respective controls. **(c, d)** Cell proliferation was analyzed by MTS assay at the indicated time points. Cell proliferation was increased by transfection with miR-1246 mimic (**c**), whereas it was decreased by transfection with miR-1246 inhibitor (**d**) when compared to their respective controls. *: $p < 0.05$ versus control, **: $p < 0.01$ versus control. RQ, relative quantification.

were found to be higher in 3-dimensional cultured cells than in 2-dimensional culture conditions. We were able to evaluate exosomes in a state mimicking the in vivo structure because patient-derived organoids mimic their original structure in 3-dimensional culture conditions. This method may contribute to new findings that have been difficult in the past research of exosomes isolated from cell lines.

Next, we investigated differences in the cellular miRNA profiles of CRC and CRA in organoids. In CRC, the expression of 4 cellular miRNAs, including miR-1246, was upregulated, while the expression of 12 cellular miRNAs, including the miR-320 and miR-200 families, was downregulated as seen in the microarray. Reportedly, miR-1246, which is elevated in CRC in comparison to the normal tissue [17], regulates apoptosis via p53 [18]. We previously reported that the expression of

the miR-320 family was downregulated in CRC when compared with CRA [19], and similar results were obtained in this study. Notably, a number of previous reports have evaluated cellular miRNA using specimens obtained from paraffin-embedded tissues or biopsy tissues. In addition, few studies have compared the expression of cellular miRNAs using organoids, as in our study. Ohsaka and Sonoyama [20] reported that the miRNA profiles in intestinal epithelial organoids resemble the intestinal epithelium in mice. It is expected that similar results can be obtained for human organoids; however, further examination is required. In addition, biopsy and paraffin-embedded tissues have a mixture of epithelial cells, fibroblasts, and immune cells. Since the organoid is composed only of epithelial cells, it is possible to prevent contamination from cells other than the epithelium. This makes organoids more suitable than biopsy

and paraffin-embedded tissues for studying cellular miRNA.

Finally, this study showed that 3 exosomal miRNAs, including miR-1246, were upregulated and the expression of 12 miRNAs was downregulated in CRC when compared to CRA. In a validation RT-PCR, only miR-1246 expression was significantly higher in CRC. Recent studies have demonstrated that exosomal miR-1246 plays an important role in cancer progression. For example, exosomal miR-1246 acts on tumor-associated macrophages, reprogramming them into a cancer-promoting state via TGF- β signaling [21]. In addition, miR-1246-enriched exosomes secreted from pancreatic cancer cells induced activation and pro-fibrogenic activities in pancreatic satellite cells [15]. Moreover, exosomal miR-1246 increases cell motility by targeting DENN/MADD domain containing 2D [22]. In this study, we show that the expression of miR-1246 is upregulated both in the cellular compartments and in exosomes. It was assumed that miR-1246 not only promotes proliferation in cells but also plays a cancer-supporting role extracellularly as exosomes in the microenvironment. In addition, serum miR-1246 is significantly higher in CRC patients than in healthy controls [23, 24]. miR-1246 is a candidate for liquid biopsy of CRC and is useful for detecting recurrence after treatment of CRC. miRNAs are considered as potential therapeutic targets. miR-34-based therapy is at phase I clinical trials for advanced solid tumors [23]. Anti-miRs targeting miR-122 is at phase II trials for treating hepatitis [24]. On similar lines, miR-1246 may be a good therapeutic target for CRC in the future. The coculture of organoids and cancer-associated fibroblasts has been reported for pancreatic cancer [25]. A coculture method could contribute to elucidating the interaction between cancer and the surrounding tissues in CRC in the future.

This study has several limitations. First, it did not include the organoids of CRC obtained from metastatic lesions. It has been reported that exosomes are also involved in distant metastasis [26], and the addition of organoids from CRC-derived metastatic lesions may potentially detect variations in exosomal miRNAs that could not be assessed in this study. Next, since the organoid is composed of a heterogeneous cell population differentiated from cancer stem cells, it is not clear what type of cell is the source of exosomal miR-1246. Furthermore, in this study, it was revealed that miR-1246 is involved in the proliferation of CRC cells; however, the detailed mechanism needs to be investigated further.

In conclusion, we successfully isolated exosomes from patient-derived colonic organoids in 3-dimensional culture. In addition, we found differences in the exosomal miRNA profiles between CRC and CRA and showed that exosomal miR-1246 expression was significantly upregulated in CRC organoids when compared to that of CRA organoids. Further elucidation of the interactions between the exosomes and the cancer microenvironment may lead to future therapeutic applications using miRNA.

Acknowledgement

We acknowledge the technical support of the Biomedical Research Core of Tohoku University Graduate School of Medicine.

Statement of Ethics

This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine (article#: 2015-4-011). All patients provided written informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was supported by JSPS KAKENHI (Grant No. JP18K15803).

Author Contributions

Hiroshi Nagai and Masatake Kuroha designed the study and wrote the initial draft of the manuscript. Yoichi Kakuta, Yoshitaka Kanazawa, and Atsushi Masamune contributed to the analysis and interpretation of data and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation and critically reviewed the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

- 1 Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut*. 2017 Apr;66(4):683–91.
- 2 Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 2011 Nov;141(5):1762–72.
- 3 Endo H, Okami J, Okuyama H, Kumagai T, Uchida J, Kondo J, et al. Spheroid culture of primary lung cancer cells with neuregulin 1/HER3 pathway activation. *J Thorac Oncol*. 2013 Feb;8(2):131–9.
- 4 Fujii M, Shimokawa M, Date S, Takano A, Matano M, Nanki K, et al. A colorectal tumor organoid library demonstrates progressive loss of niche factor requirements during tumorigenesis. *Cell Stem Cell*. 2016 Jun;18(6):827–38.
- 5 Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215–33.
- 6 Valeri N, Braconi C, Gasparini P, Murgia C, Lampis A, Paulus-Hock V, et al. MicroRNA-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. *Cancer Cell*. 2014 Apr;25(4):469–83.
- 7 Bu P, Chen KY, Chen JH, Wang L, Walters J, Shin YJ, et al. A microRNA miR-34a-regulated bimodal switch targets Notch in colon cancer stem cells. *Cell Stem Cell*. 2013 May;12(5):602–15.
- 8 Choi DS, Kim DK, Kim YK, Gho YS. Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. *Proteomics*. 2013;13(10–11):1554–71.
- 9 Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res*. 2014 Jun;24(6):766–9.
- 10 Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007 Jun;9(6):654–9.
- 11 Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res*. 2010 Dec;70(23):9621–30.
- 12 Bigagli E, Luceri C, Guasti D, Cinci L. Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: role of microRNA-210. *Cancer Biol Ther*. 2016 Aug;17(10):1–8.
- 13 Fujii M, Matano M, Nanki K, Sato T. Efficient genetic engineering of human intestinal organoids using electroporation. *Nat Protoc*. 2015 Sept;10(10):1474–85.
- 14 Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, et al. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant*. 2011 May;26(5):1474–83.
- 15 Masamune A, Yoshida N, Hamada S, Takikawa T, Nabeshima T, Shimosegawa T. Exosomes derived from pancreatic cancer cells induce activation and profibrogenic activities in pancreatic stellate cells. *Biochem Biophys Res Commun*. 2018 Jan;495(1):71–7.
- 16 Eguchi T, Sogawa C, Okusha Y, Uchibe K, Iinuma R, Ono K, et al. Organoids with cancer stem cell-like properties secrete exosomes and HSP90 in a 3D nanoenvironment. *PLoS One*. 2018 Feb;13(2):e0191109.
- 17 Piepoli A, Tavano F, Copetti M, Mazza T, Palumbo O, Panza A, et al. Mirna expression profiles identify drivers in colorectal and pancreatic cancers. *PLoS One*. 2012 Mar;7(3):e33663.
- 18 Zhang Y, Liao JM, Zeng SX, Lu H. p53 down-regulates Down syndrome-associated DYRK1A through miR-1246. *EMBO Rep*. 2011 Jun;12(8):811–7.
- 19 Tadano T, Kakuta Y, Hamada S, Shimodaira Y, Kuroha M, Kawakami Y, et al. MicroRNA-320 family is downregulated in colorectal adenoma and affects tumor proliferation by targeting CDK6. *World J Gastrointest Oncol*. 2016 Jul;8(7):532–42.
- 20 Ohsaka F, Sonoyama K. Murine intestinal organoids resemble intestinal epithelium in their microRNA profiles. *Biosci Biotechnol Biochem*. 2018 May;82(9):1560–7.
- 21 Cooks T, Pateras IS, Jenkins LM, Patel KM, Robles AI, Morris J, et al. Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246. *Nat Commun*. 2018 Feb;9(1):771.
- 22 Sakha S, Muramatsu T, Ueda K, Inazawa J. Exosomal microRNA miR-1246 induces cell motility and invasion through the regulation of DENND2D in oral squamous cell carcinoma. *Sci Rep*. 2016 Dec;6:38750.
- 23 Hong DS, Kang YK, Borad M, Sachdev J, Ejadi S, Lim HY, et al. Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br J Cancer*. 2020 Apr;122(11):1630–7.
- 24 Gebert LF, Rebhan MA, Crivelli SE, Denzler R, Stoffel M, Hall J. Miravirsin (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res*. 2014 Jan;42(1):609–21.
- 25 Seino T, Kawasaki S, Shimokawa M, Tamagawa H, Toshimitsu K, Fujii M, et al. Human pancreatic tumor organoids reveal loss of stem cell niche factor dependence during disease progression. *Cell Stem Cell*. 2018 Mar;22(3):454–e6.
- 26 Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015 Nov;527(7578):329–35.